STUDIES ON THE ACTION OF PHENYLTHIOUREA ON THE RESPIRATORY METABOLISM AND SPINNING BEHAVIOUR OF THE CYNTHIA SILKWORM

BRYN M. JONES AND RONALD S. WILSON

Department of Zoology, University of Edinburgh, Scotland, U. K.

There is now abundant proof, furnished particularly by Schneiderman and Williams (1954), and Shappirio and Williams (1957a, 1957b), of the presence of various cytochromes and of cytochrome oxidase in the tissues of the *Cecropia* silkworm.

In the larval stage, the cytochrome system is both intact and functional, and sensitive to cyanide and carbon monoxide. During the course of metamorphosis, however, there is a precipitous fall in the rate of oxygen consumption of the *Cecropia* silkworm, and, later, when diapause intervenes during the pupal stage, the presence of cytochromes *b* and *c* becomes indetectable, while cytochrome b^5 and cytochrome oxidase are present at low levels according to spectrophotometric and spectroscopic studies of individual tissues. These changes do not occur in the cytochrome system of the intersegmental muscles of the abdomen (Shappirio and Williams, 1957a, 1957b). The partial breakdown of the cytochrome system in the rest of the tissues, however, seems to be responsible for the striking decrease in respiratory metabolism.

A distinguishing feature of the metabolism of the diapause pupa is its resistance to cyanide and carbon monoxide. This is apparently due to the great excess of cytochrome oxidase relative to cytochrome *c* in this stage, and to cyanide and carbon monoxide being incapable of inhibiting this reserve cytochrome oxidase unless the oxygen tension is experimentally decreased to exceedingly low levels (Harvey and Williams, 1958a, 1958b; Kurland and Schneiderman, 1959). Previously, the cyanide- and carbon monoxide-insensitive character of the metabolism of the diapause pupa suggested the possibility that tyrosinase might be serving as a terminal oxidase in respiration instead of cytochrome oxidase. Sussman (1949, 1952), however, showed that this was not the case.

Since it has long ago been shown that cytochrome oxidase is immune to the action of various urea compounds (Grant and Krantz, 1942) and phenylthiourea (DuBois and Erway, 1946), it has been customary to use the latter compound, a copper-catalyst blocking agent, to inhibit the activity of blood phenolase to prevent the formation of toxic quinolic substances after surgical operation on insects. It was therefore of particular interest that the use of the compound for this purpose in some experiments on Cynthia silkworms seemed to depress their metabolism.

Preliminary studies on the oxygen consumption of *Cynthia* revealed significant decreases in the rate after treatment with phenylthiourea. Moreover, it was also noticed that treatment with this compound affected the nature of the cocoon spun later by the silkworm.

The difference in degree of respiratory metabolism, as shown by these preliminary results, suggested that the respiratory metabolism of *Cynthia,* though probably mediated by the usual cyanide- and carbon monoxide-sensitive cytochrome oxidase system, is sensitive to the action of a copper-catalyst inhibitor.

This investigation was therefore undertaken to elucidate further the depressant effect of phenylthiourea on respiratory metabolism, and the accompanying effect of this compound on the spinning behaviour of *Cynthia.*

Materials and Methods

The present study is based on respiratory measurements of final instar larvae and pupae of the silkworm, *Philosamia cynthia.*

Phenylthiourea was introduced into the blood of the silkworm, under CO_2 anaesthesia, either by inserting it as crystals through an incision made in the abdomen, or by injecting it in a physiological solution from a hypodermic syringe.

Both the experimental and control animals were kept in glass tubes containing a strip of moistened filter paper, and were fed on privet. They were transferred to vessels of approximately 40-cc. for use with standard Warburg manometers for oxygen-consumption measurements. Each vessel was divided into two connected well-compartments by an infolding of the base. To absorb the carbon dioxide output, a loose roll of filter paper was moistened with 0.5 to 0.7 cc. of 1.5 N sodium hydroxide and placed in one of the compartments, the silkworm being placed in the other. To protect the silkworm, another strip of filter paper was placed over the moistened roll. The silkworms only very occasionally disturbed the arrangement of filter papers.

Measurements were performed at 25° C. They were usually carried out at intervals of ten minutes over a period of half an hour or longer. The rate has been expressed as mm.^{3} oxygen per gram weight of the pupa per hour, following the procedure of Schneiderman and Williams (1953), to obviate the changeability in weight of a feeding and spinning silkworm.

Results

1. *Normal changes in oxygen consumption during metamorphosis*

The maximal rate of oxygen consumption in *Cynthia* prior to metamorphosis is in the region of 1400 mm.^{3} O_2/gm.pupal wt./hr. at 25° C. (Fig. 1).

At metamorphosis, considerable changes take place. The rate of oxygen consumption begins to fall two and a half days before the onset of spinning. It continues to fall, reaching a level of about 250 mm.^{3} O_2/hr. at the end of spinning and of 75 mm.^{3} O_2/hr. in the pupal stage.

The precipitous nature of this fall in the rate of oxygen consumption in *Cynthia* during the course of metamorphosis at 25° C. is in agreement with that recorded for *Cecropia* (Schneiderman and Williams, 1953). There is, however, an important difference with regard to the timing of the fall. Whereas in *Cecropia* the decrease begins just after the cocoon has been spun, the decrease in *Cynthia* begins two and a half days before spinning is initiated. Indeed, at the beginning of the spinning phase, in *Cynthia,* the rate of oxygen consumption has already fallen to half the maximal level.

Cynthia takes two days to construct a cocoon, the outer envelope being completed in 24 hours, and the loose intermediate layer and inner envelope in the next 24 hours.

2. Inhibitory effect of phenylthiourea on oxygen consumption

Measurements of the rate of oxygen consumption were carried out on feeding fifth instar *Cynthia* before and after treatment with phenylthiourea. The compound was inserted as crystals through an incision made in the abdomen. Though

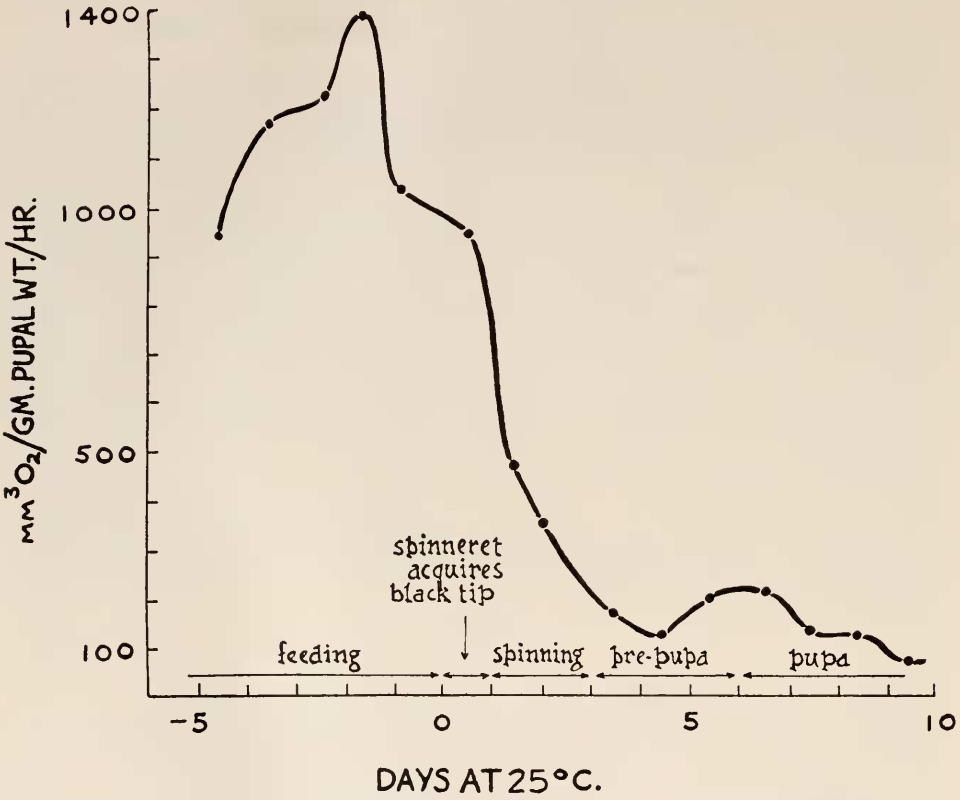

FIGURE 1. Changes in the rate of oxygen consumption at 25° C. of the *Cynthia* silkworm during metamorphosis.

it is highly insoluble in water, being taken up at about 2 mg. per cc., it dissolves readily in *Cynthia* blood. The weights of the silkworms used ranged from 3 to 4 gm.

Figure 2 shows the depressant action of the inhibitor at varying amounts on the rate of oxygen consumption. When 0.45 mg. was introduced in the way just described, there was a significant decrease in the rate of oxygen consumption of the treated silkworm. The rate subsequently increased without, however, reaching the pre-treatment level. At the onset of spinning the rate was about 650 mm.^{3} O_2/hr.

At 0.75 mg., the depressant action of the compound on the rate was more marked. The fall in the rate, however, was again followed by an increase, but this failed to reach a level above 500 mm.^{3} O_2/hr. before spinning was initiated. Moreover, silkworms treated with this amount of the compound were rendered incapable of constructing a normal cocoon, but they were not, however, prevented from pupating normally.

At 1.0 mg., there was a striking fall in the rate. Silkworms treated with this amount of the compound were, after a delay of several hours, liable to become completely immobilized. This induced quiescent state usually lasted about two

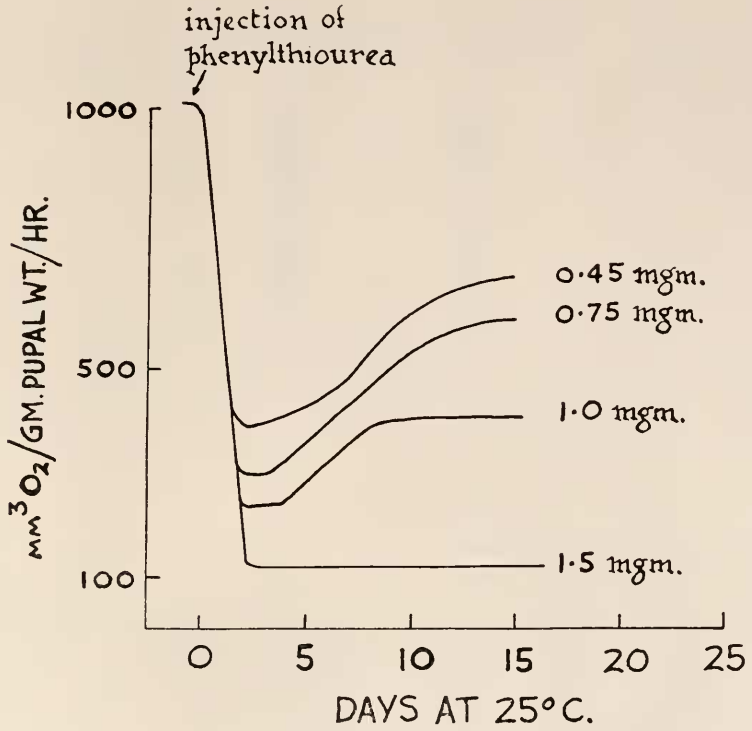

FIGURE 2. Depressant action of phenylthiourea at different concentrations on the rate of oxygen consumption of feeding fifth instar *Cynthia* larvae at 25° C.

days. This was followed by a gradual recovery which was accompanied by an increase in the rate of oxygen consumption. The rate, however, seldom reached a level above 400 mm.^{3} O_2/hr. before spinning took place. Though the usual two-day period was spent in spinning by these treated silkworms, it was observed that they failed to produce more than a limited sheet of silk.

Amounts of phenylthiourea in excess of 1.5 mg. were usually lethal to the silkworms used. They invariably brought about a precipitous fall in the respiratory rate, and so completely immobilized the treated silkworms that they seldom showed any signs of recovery.

These studies indicate that the depressant action of phenylthiourea on the rate of oxygen consumption in *Cynthia* is proportional to its concentration in the blood and tissues. When amounts of less than 0.45 mg. were introduced into the blood, they also significantly lowered the respiratory rate, and the results which indicate this are considered in a later section of this paper. Moreover, it will be apparent that the *Cynthia* silkworm seems capable of reversing the depressant action of phenylthiourea on its respiratory metabolism, provided the amount of the compound administered is less than the lethal dose.

3. *Inhibition of blood phenolase activity*

Efforts were next made to determine the inhibitory effect of phenylthiourea on the blood phenolase. Though it was unlikely that the site of the depressant action on the respiratory rate was the blood phenolase, it was nevertheless desirable to estimate the ability of this compound to inactivate a copper-catalyst which was known to be present in the blood and probably the tissues.

Phenolase (tyrosinase) is distinguished from other copper enzymes by its ability to catalyze the insertion of an hydroxyl group into monohydric phenols and the oxidation of the resulting o-diphenols to their corresponding o-quinones (Dawson and Tarpley, 1951).

It is generally accepted that the enzyme is present in the blood of insects, and that when the blood is exposed to air it changes to a brown colour owing to the enzymatic oxidation of polyphenols to form melanin-like substances. The addition of a diphenol substrate such as dihydroxyphenyl-alanine to the blood of an insect results in the rapid formation of melanin, due to the reaction being catalyzed by the phenolase. If phenylthiourea is also added, melanin is not deposited. The inference to be drawn from this is that this compound in blocking the activity of the phenolase suppresses the development of melanin.

Most inhibition studies carried out with the enzyme have been performed with substances known to form stable complexes with copper. Among the substances used, the thioureas seem to be very effective blocking agents of phenolase (DuBois and Erway, 1946). Little is known at the present time, however, as to how the copper is bound within the enzyme, or what precisely is its role in phenolase activity (Dawson and Tarpley, 1951). It is likely, however, that phenylthiourea exerts its inhibitory effect on phenolase by reacting with the copper component of this enzyme, as it does with the copper which catalyzes the oxidation of ascorbic acid (DuBois and Erway, 1946).

Dawson and Tarpley (1951) have directed attention to the fact that the oxidation of di- and polyhydric phenols can be brought about in other ways. For example, these phenols can be oxidised by hydrogen peroxide in the presence of peroxidase, and aerobically in the presence of cytochrome *c* and cytochrome oxidase. Presumably, this type of oxidation cannot be inhibited by a copper-catalyst blocking agent such as phenylthiourea which has no effect on the enzymes concerned. Copper alone, or in complex form with non-specific proteins, peptides, or amino acids, can also apparently catalyze the aerobic oxidation of these phenols. There is strong evidence, however, in support of the conclusion that in insects a blood phenolase (tyrosinase) is responsible for the formation of melanin from polyphenols (Mason, 1955).

The following tests were carried out to permit an estimation of to what extent the blood phenolase of *Cynthia* could be inhibited by the introduction of phenylthiourea. Silkworms weighing about 3 to 4 gm. were treated with amounts of either 0.45 mg. or 1 mg. of the compound by the method already described. Samples of blood were taken from these silkworms 24 hours later. To the samples was added a solution of dihydroxyphenyl-alanine so that 1 cc. of the final solution contained 1 mg. of the phenol substrate. Figure 3 illustrates samples of blood from silkworms treated or untreated previously with the inhibitor, and also samples to which the substrate has been added.

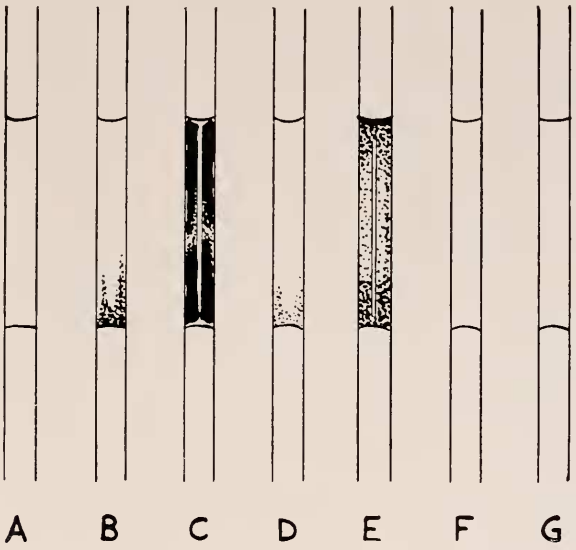

FIGURE 3. Inhibitory effect of phenylthiourea on melanin formation in *Cynthia* blood. For details see text. A, solution of diphenol substrate; B, untreated blood; C, untreated blood incubated with the diphenol; D, blood from silkworm injected 24 hours previously with 0.45 mg. of the inhibitor; E, same as D incubated with the diphenol; F, blood from silkworm injected 24 hours previously with 1 mg. of the inhibitor; G, same as F incubated with the diphenol. Drawings taken from photographs of the results of this experiment.

Though distinctions could be drawn after a few hours between the different samples according to the intensity of melanin development, they became more clearly marked at the end of 24 hours (Fig. 3). There seemed to be no further increase in the intensity of melanin deposited after about 30 hours.

It is concluded from a series of tests of the kind illustrated in Figure 3 that whereas 0.45 mg. of phenylthiourea partially blocked the activity of the blood phenolase, 1 mg. completely blocked it. Moreover, the inference to be drawn from the results of these tests is that the inhibitor is inactivating the enzyme *in vivo*.

4. *Effect of changes in the metabolic rate on cocoon spinning*

To elucidate further the effect of phenylthiourea upon the spinning behaviour of *Cynthia*, studies were next made on the depressant action of this compound on respiratory metabolism during the spinning period.

TABLE I

Relationships between the depressant action of phenylthiourea on the rate of oxygen consumption at the beginning of spinning and the types of structures spun. For details see text.

Serial no.	Mg. of agent injected 24 hr. previous to spinning	mm.^{3} O_2/gm. pupal wt./ hr.			Types of cocoon spun
		Uptake at the beginning of spinning	Uptake at the end of spinning	Range	
P 6	nil	740	210	520	Normal
P 8	0.1	700	260	440	Two-layered and closed
P 1	0.1	700	280	420	Two-layered and closed
P 4	0.3	520	280	240	Two-layered and open
P 9	0.4	500	250	250	Hammock-shape
P 7	0.5	525	265	260	One-layered and open
L 2	0.6	490	290	200	Hammock-shape
P 2	0.7	385	210	175	Hammock-shape

An important symptom of metamorphosis in *Cynthia*, as already described, is the striking fall in the rate of oxygen consumption, which begins two and a half days before the onset of spinning. The injection of phenylthiourea into silkworms during this pre-spinning period would be expected therefore further to depress the already decreasing rate of oxygen consumption. It is also reasonable to assume that this fall in the rate of respiration reflects the progress in the series of events of metamorphosis which is triggered off by the "pupation" hormone (Williams, 1947). The timing of the onset of spinning is therefore already under hormonal control, and the insertion of phenylthiourea into the blood just prior to the spinning period is unlikely to influence it.

Accordingly, the compound was injected in solution into *Cynthia* about 24 hours before spinning was due to begin. This stage was conveniently marked by

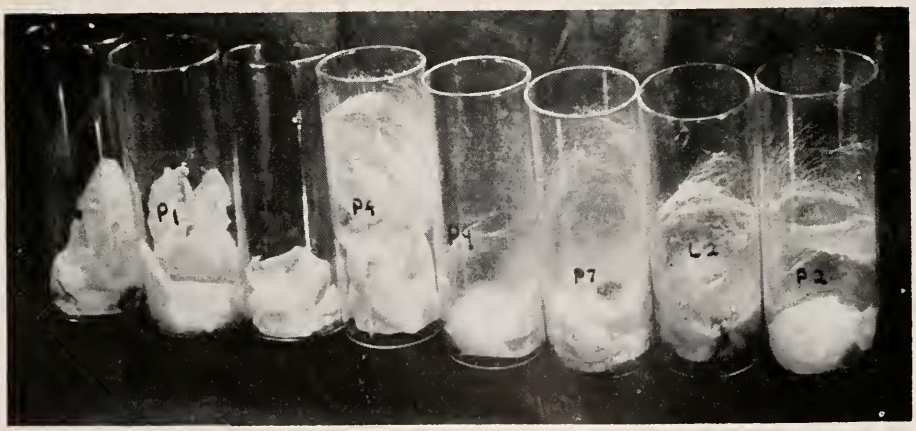

FIGURE 4. Series of structurally different cocoons spun by *Cynthia* after being injected 24 hours previously with varying amounts of phenylthiourea. From left to right in the photograph relative amounts of the inhibitor used were, nil, 0.1 mg., 0.1 mg., 0.3 mg., 0.4 mg., 0.5 mg., and 0.7 mg.

the tip of the spinneret turning black. The amounts of the compound introduced into the blood in these studies ranged from 0.1 to 0.7 mg.

The results in Table I illustrate a relationship between the depressant action of phenylthiourea on the rate of oxygen consumption at the beginning of spinning and the types of structures spun. It seems that the extent to which the rate of respiratory metabolism is decreased at the beginning of spinning determines the type of structure spun (Fig. 4).

Discussion

1. *Depressant action of phenylthiourea on respiration*

The results of this investigation show that the influence of phenylthiourea on the respiration of *Cynthia* is one of depression, which is proportional to the concentration.

During the course of normal metamorphosis in silkworms there is a striking fall in the rate of oxygen consumption. A considerable fall in the rate, however, also occurs at each moult, and it is significant that when a larva is deprived of food there is an accompanying decrease in the respiratory rate (Wilson, unpublished work). It seems, therefore, as if cessation of feeding by silkworms prior to metamorphosis may be partly responsible for the decrease in the metabolic rate.

The introduction of phenylthiourea into the blood of *Cynthia* just before the spinning phase markedly depressed the rate of oxygen consumption below the normal level. This obviates the possibility that the inhibitory effect of phenylthiourea on respiration is simply due to this compound halting feeding. The evidence instead converges in support of this compound exerting an inhibitory action upon some site in the respiratory chain of *Cynthia*. It seems unlikely, however, from previous work that the site of the depressant action is the cytochrome oxidase or succinic dehydrogenase systems (Grant and Krantz, 1942; DuBois and Erway, 1946).

Since the experimentally induced fall in the rate of oxygen consumption in *Cynthia* is accompanied by an inhibition of the blood phenolase activity, the possibility of this enzyme being implicated in some way with respiration, as suggested by Heller (1947), cannot be ruled out. Though potentially capable of serving as a terminal respiratory oxidase, it would, however, seem unnecessary for phenolase to perform this function in a silkworm in which there is an intact and functional cytochrome-cytochrome oxidase system. If one prefers to accept the possibility of phenolase serving as a terminal respiratory oxidase despite the presence of cytochrome oxidase, there then remains the further possibility that phenolase may be coupled with the oxygen which is supposed to diffuse from the tracheoles and across the short distances to the adjacent tissues.

It was suggested long ago, however, that the depressant action of urea derivatives on respiration is exerted on a part of the respiratory chain involving DPN, and not on the oxygen terminal part of the chain (Grant and Krantz, 1942). This is still believed to be the case (Slater, 1958).

Morton (1958) has recently drawn attention to the wide distribution of quinone compounds in tissues, and Slater (1958) has suggested how these compounds may be involved in oxidative phosphorylation. The evidence up till now is in favour

of this, and it is therefore tempting to suggest that if the quinones in question are derived from quinol compounds only in the presence of an active phenolase-type enzyme, then blocking this catalyst would be expected to have an inhibitory effect on oxidative phosphorylation. To speculate further than this is unlikely to be of much value because there are still many gaps in our knowledge about the processes involved in oxidative phosphorylation.

2. Relation of metabolic rate to spinning behaviour

It will be seen from the results shown in Table I that the differences between the cocoons spun by *Cynthia* after treatment with varying amounts of phenylthiourea are qualitative. This obviates the results being attributed to a general muscular debility. Moreover, during the spinning period the treated silkworms were as active as the controls. This recalls the observation of Van der Kloot and Williams (1954) that *Cecropia* retains its normal activity when exposed to carbon monoxide yet at the same time is rendered incapable of spinning a cocoon.

The results of the present work show that the type of cocoon spun depends on the extent that the rate of oxygen consumption is depressed at the beginning of the spinning phase by phenylthiourea. Since the cocoon is the end-result of a complicated pattern of neuromuscular activity, any deviation from normal in the structure of the cocoon may be taken as reflecting a change of behaviour. It is therefore reasonable to conclude that quantitative differences in the metabolic rate are coupled in the silkworm with differences in behaviour which are qualitative. It would be interesting to learn to what extent this principle can be extended to other forms of behaviour, whether instinctive or otherwise.

Summary

1. A symptom of the metamorphosis of *Cynthia* is that the precipitous fall in the rate of oxygen consumption begins two and a half days before the spinning period.

2. The rate of oxygen consumption is reduced to half the maximal rate of 1400 mm.^{3} O_2/gm. pupal wt./hr. when spinning begins. It continues to fall throughout the spinning phase and reaches a level of 75 mm.^{3} O_2/hr. in the early pupal stage.

3. Phenylthiourea has a pronounced depressant action on the rate of oxygen consumption, the depression being proportional to the amount of this compound introduced into the blood.

4. The decrease in respiration brought about by phenylthiourea coincides with the inhibition of blood phenolase activity. Possible sites of the depressant action of phenylthiourea on respiration are discussed.

5. When the rate of oxygen consumption is depressed to various levels at the beginning of spinning a series of qualitatively different cocoons is produced.

6. It is concluded that the pattern of spinning behaviour is delicately tuned to the metabolic rate.

Literature Cited

Dawson, C. R., and W. B. Tarpley, 1951. Copper oxidases. *In:* The Enzymes, *2*, Pt. 1. (J. B. Sumner and K. Myrback, editors) New York, Academic Press: 454–498.

DuBois, K. P., and W. F. Erway, 1946. Studies on the mechanism of action of thiourea and related compounds. II. Inhibition of oxidative enzymes and oxidations catalyzed by copper. *J. Biol. Chem.*, **165**: 711–721.

Grant, W. C., and J. C. Krantz, 1942. The mechanism of action of certain urea derivatives on normal and tumour tissue. *Cancer Res.*, **2**: 833–836.

Harvey, W. R., and C. M. Williams, 1958a. Physiology of insect diapause. XI. Cyanide sensitivity of the heartbeat of the Cecropia silkworm, with special reference to the anaerobic capacity of the heart. *Biol. Bull.*, **114**: 23–35.

Harvey, W. R., and C. M. Williams, 1958b. Physiology of insect diapause. XII. The mechanism of carbon monoxide-sensitivity and -insensitivity during the pupal diapause of the Cecropia silkworm. *Biol. Bull.*, **114**: 36–53.

Heller, J., 1947. Metabolism of insect metamorphosis. *Abstr. 17th Intern. Physiol. Cong. Oxford:* 274–276.

Kurland, C. G., and H. A. Schneiderman, 1959. The respiratory enzymes of diapausing silkworm pupae: A new interpretation of carbon monoxide-insensitive respiration. *Biol. Bull.*, **116**: 136–161.

Mason, H. S., 1955. Phenolase complex. *Advances in Enzymol.*, **16**: 105–184.

Morton, R. A., 1958. Ubiquinone. *Nature*, **182**: 1764–1767.